

Preliminary Amendment

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Applicant(s): Charles W. FORD et al.

Serial No.: 09/600,392

Filed: 14 July 2000

Int'l Filing Date: 12 January 1999

For: AN AUTOREGULATORY SYSTEM FOR VALIDATING MICROBIAL GENES AS POSSIBLE
ANTIMICROBIAL TARGETS USING A TETRACYCLINE-CONTROLLABLE ELEMENT

Please replace the paragraph at page 1, line 24, to page 2, line 4, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

A2
With the availability of whole microbial genome sequences, there are now many previously unknown and uncharacterized genes available which may turn out to be essential. The conventional approach for testing if a gene is essential is to attempt making a construct of that organism where the test gene is deleted or inactivated. If the organism can survive with the gene deleted or inactivated, the gene is not considered essential. For example, see Strandén, A. M., Ehlert, K., Labischinski, H., and Berger-Bachi, B., 1997, *J. Bacteriol.* 179:9-16. However, failure to create a mutant organism with an inactivated or deleted gene does not always mean that the gene is essential. For example, see Okada, K., Minehira, M., Zhu, X., Suzuki, K., Nakagawa, T., Matsuda, H., and Kawamukai, M., 1997, *J. Bacteriol.* 179:3058-3060. This negative proof for a conclusion may not always be valid. There may be other reasons why the gene deletion or inactivation could not be made.

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Please replace the paragraph at page 4, lines 17-25, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

A3
A meaningful difference between the two groups of animals being tested is a mathematically significant difference in the survival rates or the levels of microbes, or levels of infection present in the mammals. The meaningful difference between the two groups of animals is a mathematically significant difference in the survival rates of the groups of animals. The significant difference in the survival rates of the groups of animals shows that animals exposed to tetracycline have poorer health, higher rates of infection, lower survival or higher levels of microbes than animals not exposed to tetracycline. The animals can be mammals, preferably mice or other rodents.

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Please replace the paragraph at page 5, lines 7-22, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

al This isolated DNA molecule can have a polynucleotide sequence encoding a reporter gene operably linked to the TCE. The reporter gene can be beta-lactamase. In some cases at least one prokaryotic transcription terminator polynucleotide sequence is positioned between the RE and the TCE. The DNA can also have a polynucleotide sequence encoding a prokaryotic tetracycline resistance protein operably linked to a prokaryotic transcription promoter polynucleotide sequence positioned between the RE and the TCE. The tetracycline resistance protein can be derived from the *Staphylococcus aureus tetM* gene. The DNA can have a polynucleotide sequence encoding a prokaryotic tetracycline repressor protein operably linked to a tetracycline-controllable prokaryotic transcription promoter polynucleotide sequence positioned between the RE and the TCE. The tetracycline repressor may be a Tn10 transposon, derived from a Tet repressor. Sequences of Tn10 transposons are disclosed herein. Associated vectors and cells, especially prokaryotic host cells, are described. The DNA has various recombining elements and tetracycline-controllable elements, reporter genes like beta-lactamase whose sequences may be selected from the sequence listing.

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Please replace the paragraph at page 5, line 27, to page 6, line 4, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

Q5 The tetracycline resistance protein can be derived from the *Staphylococcus aureus tetM* gene or from various sequences provided. The tetracycline repressor may be a *tetR* gene derived from the Tn10 transposon, and several sequences are provided. At least one prokaryotic transcription terminator sequence can be positioned between the tetracycline-controllable element and one or more recombining elements. A prokaryotic tetracycline resistance protein can be operably linked to a transcription promoter polynucleotide sequence. A polynucleotide sequence encoding a tetracycline repressor protein can be operably linked to a transcription promoter polynucleotide sequence. The DNA described here can be made into a form suitable for transformation of a host cell.

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Please replace the paragraph at page 6, lines 5-26, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

Ab The invention further comprises another different type of isolated DNA molecule for integrating a heterologous polynucleotide sequence at a pre-determined location in a prokaryotic cell. This other type of DNA can be described as an isolated DNA molecule for integrating a polynucleotide sequence including tetracycline-controllable elements (TCE) at a pre-determined location in a target DNA molecule, the isolated DNA molecule comprising the following DNA elements fused in sequence: a) a first prokaryotic transcription terminator polynucleotide sequence; b) a second prokaryotic transcription terminator polynucleotide sequence; c) a polynucleotide sequence encoding a prokaryotic tetracycline resistance protein; d) a polynucleotide sequence encoding a prokaryotic repressor protein; e) a first tetracycline-controllable prokaryotic transcription promoter polynucleotide sequence; f) a second tetracycline-controllable prokaryotic transcription promoter polynucleotide sequence; and g) a polynucleotide sequence encoding a reporter protein; the isolated DNA molecule comprising a polynucleotide sequence including the TCE flanked at the end opposite the polynucleotide sequence encoding the reporter protein by additional polynucleotide sequences of sufficient length for homologous recombination between the isolated DNA molecule and the target DNA molecule at a pre-determined location. All of the modifications described above can be applied to the DNA molecule described in this paragraph. This DNA molecule may also be described as a DNA cassette, it may also be called an RDC. Note an RDC does not have to be on a single cassette, the elements of an RDC can be fashioned in many different ways. Elements of the RDC can even be taken from the microbe itself.

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Please replace the paragraph at page 6, lines 27-31, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

A7
Finally, this system is described in detail with bacterial organisms, but it can also be adapted to other types of organisms. When the system is used with a virus, eukaryote or yeast, the transcription promoters and structural genes should be modified in a manner apparent to one skilled in the art that would make the promoters and genes active in that organism.

Please replace the paragraph at page 8, lines 8-16, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

A8
Figure 5, SEQ. ID. NO. 37, is the nucleotide sequence of the synthetic DNA fragment of the regulatory cassette containing two diverging transcriptional promoters with *tetO* sequences. The nucleotides in bold letters comprise recognition sequences for the restriction endonucleases indicated above in italics. Capitalized nucleotides on both DNA strands represent *tetO* sequences, putative binding sites for the tet repressor protein in the absence of tetracycline. The -35 and -10 regions of the *tet* promoter (P_{tet}) and *xyl* promoter (P_{xyl}) are underlined and overlined, respectively. The capitalized ATG on the bottom strand indicates the start codon of the *tetR* open reading frame.

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Please replace the paragraph at page 9, lines 25-27, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

99 C when followed by a number refers to temperature in degrees celsius. The C may be followed by a slash"/" and a number, or the C may be followed by a superscript "°" and a number, e.g. C/37 or C°37.

Please replace the paragraph at page 9, lines 28-29, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

910 Beta-lactamase or β -lactamase is a reporter gene and protein. It is further described below.

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Please replace the paragraph at page 13, lines 19-29, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

all
The tetracycline-controllable element (TCE) system in the example shown here is based on regulatory elements of a tetracycline-resistance operon. Tn10 is a transposon with a tetracycline-regulatory system. Tn10 is described in Hillen & Wissmann, "Tet repressor-*tet* operator interaction," in *Protein-Nucleic Acid Interaction*, Saeger and Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp.143-162), incorporated by reference into this document. Transcription of resistance-mediating genes within Tn10 is negatively regulated by a tetracycline repressor (TetR). In the presence of tetracycline or a tetracycline analogue, TetR does not bind to its operators located within the promoter region of the operon, allowing transcription. Promoters operably fused to tetracycline operator (*tetO*) sequences are virtually silent in the presence of TetR and low concentrations of tetracycline.

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Please replace the paragraph at page 13, line 30, to page 14, line 3, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

A12 The specificity of the Tet R for its operator sequence (Hillen & Wissmann, "Tet repressor-*tet* operator interaction," in *Protein-Nucleic Acid Interaction*, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp.143-162) as well as the high affinity of tetracycline for TetR (Takahashi et al., *J. Mol. Biol.*, 187:341-348 (1986)) and the well-studied chemical and physiological properties of tetracyclines constitute a basis for an inducible expression system in prokaryotic cells.

Please replace the paragraph at page 15, lines 15-22, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

A13 Option I. The TCE alone; the TCE ligated to tetR, tetM and BlaZ; or the full RDC, can be inserted into the chromosome. Recombining elements (RE) flanking the inserted DNA should be designed to have enough sequence identity with the host chromosomal DNA to allow homologous recombination into the chromosome. The RE sequences are designed to target insertion so that the cassette is between the target gene and its endogenous transcription promoter sequences. In this way, the natural controlling sequences are removed from the target gene, and the target gene expression is controlled by the TCE as inserted or the TCE as part of the RDC.

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Please replace the paragraph at page 17, lines 1-11, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

A14 A preferred embodiment of the invention relates to an isolated DNA molecule, or DNA cassette, for integrating a heterologous polynucleotide sequence at a pre-determined location in a microbial chromosome to operably control an endogenous prokaryotic gene or as an extrachromosomal element cloned such that it operably controls a functional copy of the targeted gene, the DNA molecule comprising a tetracycline controllable element (TCE) where the TCE comprises a tetracycline-controllable prokaryotic transcription promoter. For integration into the microbial chromosome, the TCE polynucleotide sequence is flanked at its 5' end, and optionally and the 3' end, by a recombining element (RE), where the RE comprises additional polynucleotide sequences of sufficient length for homologous recombination between the isolated DNA molecule and the microbial chromosome.

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Please replace the paragraph at page 19, lines 11-19, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

Q15 A DNA cassette is constructed for introduction into *S. aureus* either by homologous recombination into the *S. aureus* chromosome at a specific site by Campbell-type recombination, see Campbell, A., 1962, *Advan. Genet.*, 11:101-145, incorporated into this document by reference, or on an autonomously replicating plasmid. For chromosomal integration, this DNA contains a region at one or both ends homologous to regions of the *S. aureus* chromosomal DNA. The rest of the construct contains a recombinant DNA cassette as illustrated in **Fig. 1**. On an autonomously regulated plasmid, the recombinant DNA cassette in Figure 1 would contain DNA encoding a *S. aureus* gene.

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Please replace the paragraph at page 19, line 20, to page 20, line 16, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

A16

The first element of this cassette contains two transcription terminators, which are designed to prevent transcriptional read-through from the chromosomal DNA into this insert as well as transcriptional read-through from the cassette into the chromosome. These are followed by a *S. aureus* gene conferring resistance to tetracycline, *tetM*. This gene was chosen because the mechanism of resistance does not appear to change the structure or concentration of tetracycline in the cell, rather it appears to provide an alternative elongation factor which is resistant to the tetracycline in translation, see Nesin, M., Svec, P., Lupski, J. R., Godson, G. N., Kreiswirth, B., Kornblum, J. and Projan, S. J., *Antimicrob. Agents Chemother.*, 1990, 34:2273-2276, incorporated into this document by reference. This gene is transcribed from left to right as shown in **Figure 1**. Alternatively, *tetM* could be incorporated somewhere else in the chromosome of *S. aureus* to provide a background strain useful for a number of targeted gene tests. The gene encoding *E. coli* tet repressor, *tetR*, see Postle, K., Nguyen, T. T., and Bertrand, K. P., *Nuc. Acids Res.*, 1984, 12:4849-4863, incorporated into this document by reference, is transcribed as an operon with *tetM* from an adjacent promoter on the region containing two diverging promoters (P_{tet} and P_{xyl}) and two tetracycline operator sequences (*tetO*). The tet repressor protein binds *tetO* sequences in the absence of tetracycline, preventing transcription from P_{xyl} . In the presence of tetracycline, tet repressor binds tetracycline and not *tetO* sequences, allowing transcription from P_{xyl} . The strong *B. subtilis* promoter, P_{xyl} , signals initiation of transcription to the right as drawn in **Figure 1**, allowing transcription of *S. aureus* *BlaZ* encoding beta-lactamase, an assayable marker gene which confers resistance to ampicillin, see Wang, P. Z. and Novick, R. P., 1987, *J.*

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Serial No.: 09/600,392

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For: AN AUTOREGULATORY SYSTEM FOR VALIDATING MICROBIAL GENES AS POSSIBLE
ANTIMICROBIAL TARGETS USING A TETRACYCLINE-CONTROLLABLE ELEMENT

Please replace the paragraph at page 21, lines 24-32, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

a18
The structural gene of *S. aureus tetM* (Genbank accession number M21136) was amplified by PCR as described above, using primers CLQ463 and CLQ464 listed in Table I. These primers add unique recognition sites for the restriction enzymes BamHI and XmaI, respectively. The template for amplification was provided by Serban Iordanescu (Public Health Research Institute, NY), plasmid pRN6880, and is derived from the plasmids published by Nesin, M., Svec, P., Lupski, J. R., Godson, G. N., Kreiswirth, B., Kornblum, J. and Projan, S. J., *Antimicrob. Agents Chemother.*, 1990, 34:2273-2276. **Figure 3** shows the polynucleotide sequence of this DNA fragment.

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Please replace the paragraph at page 23, lines 13-30, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

a19
After all the PCR and synthetic DNA elements are assembled into a single cassette, the DNA cassette is ligated in a *S. aureus* plasmid. For those constructs designed to integrate into the chromosome, the cassette is also ligated to insertion-directing sequences made of homologous chromosomal DNA. The plasmid is passaged through *S. aureus* RN4220, see Peng H., Novick, R. P., Kreiswirth, B., Kornblum, J. and Schlievert, P., 1988, *J. Bacteriol.*, 170, 4365-4372, incorporated into this document by reference, a restriction minus, modification positive strain. Plasmid DNA purified from RN4220 is modified by native *S. aureus* DNA modification enzymes and is more readily transformed into pathogenic *S. aureus* strains that have wild-type DNA restriction systems, see Iordanescu, S. and Surdeanu, M., 1976, *J. Gen. Microbiol.*, 96, 277-281, incorporated into this document by reference. Insert DNA released by EcoRI restriction enzyme digestions is purified and circularized. This DNA is transformed into a pathogenic *S. aureus* strain, selecting for tetracycline resistance. Because the insert DNA does not have an origin of replication, it should not be maintained as an autonomous plasmid, and growth on tetracycline selects for recombinants where the cassette has been inserted into the chromosome. Southern Blots or PCR analysis are used to verify that the desired recombination event has occurred.

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Please replace the paragraph at page 25, line 17, to page 26, line 16, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

Q20
In the second example, the validity of integrating the cassette into the chromosome is tested by controlling the regulation of a gene assumed to be essential for *S. aureus* growth: the gene encoding elongation factor Tu (EF-Tu). EF-Tu is required for protein translation and is a proven target for antibiotics. (Selva, E., Montanini, N., Stella, S., Soffientini, A., Gastaldo, L. and Denaro, M., 1997, *J. Antibiot. Tokyo* 50, 22-26, incorporated by reference.) Primers CLQ455 and CLQ456 from **Table 1** were used to PCR amplify one 320 base pair fragment from *S. aureus* chromosomal DNA corresponding to a region of DNA just upstream from the EF-Tu structural gene and including the 3' end of the structural gene for elongation factor G (**Figure 7a**). A second fragment, PCR amplified using primers CLQ505 and CLQ506 from **Table 1**, corresponds to a region overlapping the 5' end of the EF-Tu structural gene (**Figure 7b**). The insertional DNA cassette was constructed by ligating these fragments to element 1 and element 5a, respectively. When this DNA fragment is used to transform *S. aureus* cells, the fragments direct recombination of the insert into the chromosome about 20 bp before the putative ribosome binding site for the EF-Tu gene in the *S. aureus* chromosome. Insertion of the DNA fragment in the chromosome is selected by growth on tetracycline and ampicillin. Recombination into the desired site can be confirmed by Southern Blot or PCR analysis of chromosomal DNA. This example serves as a positive control for the regulatory system. If the regulatory elements function as predicted, the presence of tetracycline will allow transcription of the beta-lactamase marker gene as well as EF-Tu, and the cells will grow on media with or without ampicillin. In the absence of tetracycline, the tet repressor should bind the promoter, preventing transcription of beta-lactamase and

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EF-Tu. In this case, the cells would not be expected to survive in the presence or absence of ampicillin because EF-Tu is expected to be essential. If they do survive, levels of beta-lactamase produced by these cells can be measured at different tetracycline concentrations to determine the level of repression achieved with the tet repressor. As long as there is some repression, this control can be tested in the animal infection to see if an infection established by these cells in the presence of tetracycline can persist in the absence of tetracycline. This is an indicator for how sensitive the system will be in testing target genes.

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Please replace the paragraph at page 26, line 18, to page 27, line 14, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

C21
In the third example, the DNA cassette is constructed to allow testing of the *S. aureus femA* gene (Genbank accession number M23918). Elements 1, 2, 3, 4 and 5 are the same as the elements in Example 2. These elements were fused to two pieces of DNA corresponding to *S. aureus* chromosomal DNA around the *femA* structural gene. This gene has been identified as a virulence factor: insertional inactivations of the gene reduce the virulence of a *S. aureus* pathogen (Mei, J., Nourbakhsh, F, Ford, C.W., Holden, D.W., *Mol. Microbiol.*, Oct. 1997, 26(2):399-407). Primers CLQ451 and CLQ452 from **Table 1** were used to amplify one 369 base pair fragment of *S. aureus* chromosomal DNA just upstream from the *femA* structural gene and including the 3' end of *trpA* (**Figure 8a**). Primers CLQ501 and CLQ502 were used to amplify a second fragment of *S. aureus* chromosomal DNA overlapping the 5' end of the *femA* structural gene (**Figure 8b**). Ligation of the first fragment to element 1 in the insertional DNA cassette and the second fragment to element 5a directs recombination of the insert into the chromosome about 25 bp before the putative ribosome binding site of *femA* in the *S. aureus* chromosome when cells are transformed with this construct. Again, insertion of the DNA fragment in the chromosome is selected by growth on tetracycline and ampicillin. Recombination into the desired site is confirmed by Southern Blot or PCR analysis of genomic DNA isolated from the recombinant cells. Variation in repression of beta-lactamase expression in the presence or absence of tetracycline is expected to be similar for that seen in Example 2. However, *femA* is reportedly not an essential gene for growth of the cells *in vitro* (Strandén, A. M., Ehler, K., Labischinski, H., and Berger-Bachi, B., 1997, *J. Bacteriol.*, 179:9-16), so these recombinant cells would be expected to grow